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<p>During the current year of this award, work continued on a systematic analysis of the abundance of estrogen receptor mRNA splicing variants in several human breast tumor specimens. Plasmid vectors have been developed to allow the expression of these variant receptor proteins and to enable characterization of their function. Results have been obtained with respect to stability of expression, DNA-binding activity, subcellular localization, and transcriptional activity of each of six exon-skipped receptor variants. Work continues on analyzing these variants and determining their prevalence in breast tumor cells.</p> <p>Due to a change in the institutional affiliation of the PI (Dr. R.J. Miksicek), the performance site for this project was changed through a negotiated research subcontract between The Research Foundation of SUNY and Michigan State University. Approval for this request from the US Army MRMC was received during the current award year.</p>					
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FOREWORD

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INTRODUCTION

Title: Splicing Variants of the Estrogen Receptor in Breast Cancer
 ID No.: DAMD1794J4372
 PI: Richard J. Miksicek
 Period: 09/30/95 through 09/30/96

Preface

This USAMRDC Breast Cancer Research Award was made to Richard J. Miksicek, who currently holds a primary appointment as Assistant Professor of Physiology at Michigan State University (MSU). This annual report covers the second year of this award, through 09/30/96.

During the first year of this award, the P.I. (Richard Miksicek) accepted a new position (effective 07/01/95) in the Department of Physiology at MSU, retaining an appointment as Adjunct Assistant Professor in the Department of Pharmacological Sciences of SUNY @ Stony Brook. At that time, permission was requested from the US Army MRDC through the awardee institution (The Research Foundation of SUNY) to change the site of performance of this project from SUNY @ Stony Brook to MSU by establishing a research subcontract between these institutions. This research subcontract stipulated that Dr. Sandra Haslam (Professor of Physiology and Director of the MSU Breast Cancer Program) be named P.I. of record for the MSU subgrant with Dr. Miksicek serving as Principal Co-Investigator with primary responsibility for the conduct of research. This subcontract was accepted by both institutions on 03/28/96 to become effective retroactively to 07/01/95. Predictably, progress on this project was substantially delayed between 07/01/95 and 03/28/96 pending negotiation of the research subcontract and release of research funds at MSU to the PI and Co-PI for the payment of salaries, the purchase of supplies, and cost of research services. During this period, the investigator's laboratory was re-established at MSU and the following research staff were recruited to resume work on this project:

Key Personnel:

<u>Name</u>	<u>Degree</u>	<u>Role</u>	<u>Effort</u>	<u>Starting Date</u>
MIKSICEK, Richard	Ph.D.	Principal Co-Investigator	25%	09/94
BOLLIG, Aliccia	B.S.	Graduate Research Asst.	100%	10/95
MORRISON, Mary	M.S.	Research Asst.	15%	02/96
ANKRAPP, David	Ph.D.	Postdoctoral Research Fellow	100%	07/96

Dr. Miksicek receives partial summer salary from this award. Ms. Bollig and Dr. Anrapp currently draw full salary support from this award. Ms. Morrison is supported from a non-overlapping award, but she provides significant technical support to this project in the form of cell culture, media preparation, laboratory administration, antibody preparation, and technical assistance. In addition, this award provides supplemental salary for a part-time work study student.

Project Scope

Recent work from this and other laboratories have shown that human breast tumors and tumor-derived cell lines contain a mixed population of estrogen receptor (ER) messenger RNAs (mRNAs), representing both correctly and aberrantly processed transcripts (1-4). The aberrant ER mRNAs are predicted to give rise to structurally altered receptor molecules able to interfere with the

normal estrogen response pathway present in breast tumor cells. These variants may provide a molecular explanation for the loss of estrogen responsiveness or the acquisition of resistance to tamoxifen and other estrogen antagonists (5), thereby reducing the effectiveness of hormonal adjuvant therapy of breast cancer. We received this award to undertake a detailed analysis of splicing variants of ER mRNA in selected human breast tumor specimens and to characterize the functional activity of these variants in a model cell culture system. Additionally, we are developing techniques and reagents (both molecular and immunological) to enable further investigation of the clinical significance of these ER splicing variants.

The approach being used in this project involves amplification of ER complementary DNAs (cDNAs) using the polymerase chain reaction (PCR), followed by sequence analysis of the resulting clones. In parallel, plasmid vectors have been developed to enable expression and functional characterization of each ER splicing variant in a cell culture transfection system. The original statement of work for this project included the following specific tasks:

Task 1, Identification of ER mRNA variants in breast tissue (months 1-24):

- a. Preparation of oligonucleotide primers for the analysis of ER mRNA splicing variants and optimization of PCR amplification conditions.
- b. PCR amplification of ER cDNAs prepared from tamoxifen-resistant tumors.
- c. PCR amplification of Progesterone Receptor cDNAs prepared from tamoxifen-resistant tumors.
- d. PCR amplification of ER cDNAs prepared from normal human breast tissue.

Task 2, Functional analysis of ER mRNA splicing variants (months 18-48):

- a. Construction of ER expression plasmids harboring variant ER cDNAs.
- b. Analysis of the transcriptional stimulatory activity or inhibitory activity of ER splicing variants by transient transfection.
- c. Production and characterization of normal human mammary epithelial cell lines that stably express the constitutive ER Δ E5 variant.

EXPERIMENTAL METHODS

PCR amplification of ER mRNA splicing variants.

As previously reported, twenty seven oligodeoxynucleotides were purchased from Genosys Biotechnology, Inc. (The Woodlands, TX). These oligos were designed in pairs, with sequences overlapping each of the seven splice junctions contained within the protein coding region of the ER mRNA. One member of each pair contains transcribed (upper strand) sequences corresponding precisely to correctly processed (wt) ER mRNA, while the second primer contains one to three nucleotides of divergent sequence at its 3'-terminus, corresponding to the novel junction of an exon-skipped splice. When combined with an appropriate lower strand primer containing downstream sequences, we have been able to demonstrate that these primer pairs can be used in three-primer reactions to independently amplify sequences from splicing variants in the presence of wt ER mRNA. For each primer set, titration conditions have been completed to optimize for high-fidelity priming of variant-specific primers exclusively to the cognate variant templates, but not to wt templates. Yields of PCR products are being quantitated by the inclusion of 10 μ Ci of $\alpha^{32}\text{P}$ dGTP in each PCR reaction, followed by scanning densitometry of autoradiographs or phosphorimager analysis.

Construction of plasmids for efficient expression of individual ER mRNA splicing variants.

As reviewed in Fuqua *et al.* (2), Miksicek (6), and more recently by Sluyser (7), a population of variant ER cDNAs have now been cloned and sequenced from a number of breast tumors and established tumor cell lines. The most common variants harbor a precise deletion of one of the internal exons that contribute to the structure of the mature ER protein. These variants will be referred to as ER Δ E2 through ER Δ E7, where the deleted exon is indicated numerically. To further characterize the residual function maintained by each of these receptor variants, plasmids have been constructed to support their individual expression following transient transfection into cultured cells. These plasmids represent derivatives of pCMV4 (8) and pcDNA3.1 (InVitrogen Corp., San Diego, CA), which we have shown can support high levels of receptor expression in a variety of mammalian cell lines, including Cos7 (9). Derivatives expressing each of the exon-deleted ER splicing variants (ER Δ E2, ER Δ E3, ER Δ E4, ER Δ E5, ER Δ E6, and ER Δ E7) have been completed. Plasmids expressing ER Δ E4, ER Δ E5, and ER Δ E6 were generated using synthetic oligonucleotides to construct the variant splice junctions within a wt ER cDNA expression plasmid, while the remaining plasmids involved the use of flanking restriction sites to shuttle cloned mutations (1) into the appropriate expression vectors.

Characterization of the functional activity of ER splicing variants.

To measure the ability of ER splicing variants to stimulate gene expression, transient transfection assays were performed as previously described (1). Briefly, this assay utilizes a calcium phosphate-mediated transfection procedure to introduce plasmids harboring variant ER cDNAs into HeLa cells along with an estrogen-responsive reporter plasmid (pERE-TK-CAT). More recently, we have relied increasingly upon electroporation as the transfection technique of choice, due to the greater reproducibility of transfection conditions that can be obtained using electroporation. Transcriptional activity of receptor variants in the presence or absence of hormone is assessed by measuring the activity of chloramphenicol acetyltransferase (CAT) enzyme produced in cultures of transfected cells. Levels of ER expression are being assessed by western blot analysis using an ER-specific monoclonal antibody (Mab-17) that was previously developed in this laboratory (10). DNA-binding activity was measured for each splicing variant in a gel mobility shift assay using 25-100 μ g of soluble protein from 0.4 M KCl extracts prepared from transiently transfected Cos7 cells as previously described (10). Subcellular distribution of ER variants compared with the wild-type receptor (wt ER) has been determined by indirect immunofluorescence analysis using Mab-17 as described in Neff *et al.* (10).

RESULTS

Three-Primer PCR amplification to quantitatively compare variant ER mRNA levels.

By careful attention to primer annealing conditions (time and temperature) and primer elongation times used during PCR amplification, conditions have been identified to use three-primer PCR for the simultaneous amplification of ER mRNA representing wt and variant transcripts in a single reaction tube. This technique employs a pair of 3'-mismatched primers, one of which directs PCR amplification exclusively from the wt ER cDNA, while the second primer is specific for a selected ER variant. The advantage of this technique is that it permits a direct determination of the ratio of correctly to aberrantly processed mRNA for each of the seven splice junctions of the mature ER mRNA, without the need for confirmatory sequencing to assign the identity of each shortened (exon-skipped or internally deleted) PCR product. For example, in the model PCR reactions shown in Fig. 1, specificity of the exon 7-deleted primer (E6U Δ E7,

Appendix A) for its cognate ER Δ E7 template was achieved in a PCR cycle involving a 15 second denaturation step at 95 °C, a relatively stringent 30 second annealing step at 58 °C, followed by a 60 second extension step at 72 °C. As can be seen, the exon 7-deleted primer supported efficient PCR amplification of an ER Δ E7 template (lane 6-8) without supporting any measurable amplification of a wt ER cDNA template (lane 9). The converse was also true for matched wt ER primer (E6Uwt) which amplified only the wt template (lanes 2-4) without recognizing the mutated ER Δ E7 template (lane 5). These and similar results using primer sets designed for the remaining splice junctions demonstrate the feasibility of performing three-primer reactions to selectively amplify both correctly and aberrantly spliced ER cDNA sequences in a single tube reaction. This situation has held for primer pairs designed to examine the aberrant ER Δ E2, ER Δ E4, ER Δ E5, and ER Δ E7 splices. An appropriate, cloned template has not been available to test the ER Δ E6-specific primer, since this splicing variant has not yet been reported to occur in breast cell lines or tumor samples. A secondary priming site was observed in model PCR experiments using the ER Δ E3 primer, necessitating that a modified primer be designed and synthesized for the detection of this splicing variant.

Experiments are underway using RNA extracted from MCF7 cells and from a frozen sample of human ductal carcinoma *in situ*, to quantitatively compare correctly and aberrantly spliced transcripts in these tissue sources. For these and future experiments, total RNA extractions are being performed using Trisoyl (GibcoBRL), followed by enrichment of polyadenylated RNA using Oligo dT cellulose. These RNAs will be used to selectively generate a population of ER-specific cDNAs by reverse transcription from a lower-strand ER oligonucleotide, designed to prime cDNA synthesis starting within exon 8, just downstream of the coding region of the ER protein. Standard curves are currently being constructed to enable the extrapolation of variant-to-wt template ratios from control PCR reactions primed with known amounts of cloned cDNAs corresponding to wt ER and each of its splicing variants.

Once we have adequately documented the quantitative potential of this approach using the samples described above, we will broaden our efforts to include additional breast cell lines and tumor samples. Following our original specific aims, our analysis is intended to focus on breast tumors with a high likelihood of expressing variant receptors, such as tumors that have relapsed during tamoxifen therapy and that maintain their ER-positive status despite developing resistance to tamoxifen.

Characterization of the functional activity of ER splicing variants.

With the exception of ERAE5, HeLa cell cotransfection experiments designed to assess the transcriptional activity of individually expressed splicing variants have failed to demonstrate any ability of these variant receptors to support gene activation through an estrogen response element

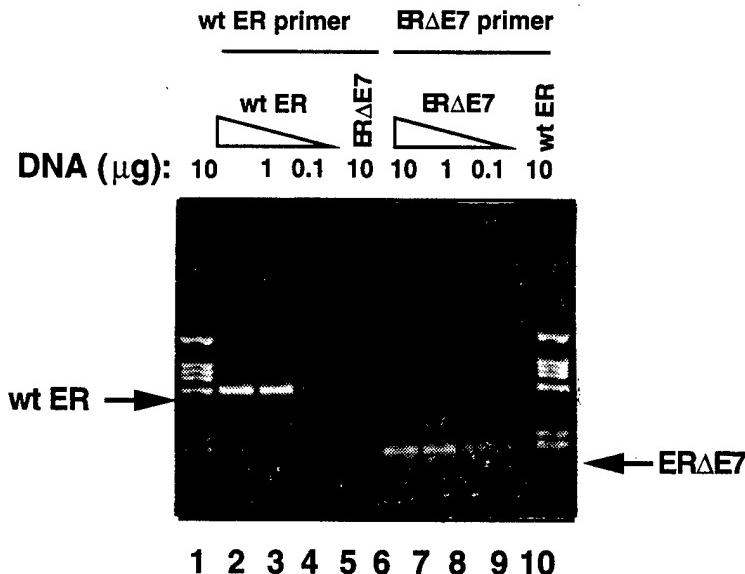


Fig. 1. PCR amplification using 3'-mismatched primers.

(ERE). The presence or absence of 1 nM estradiol has no influence on the activity of these receptors (data not shown). These data agree with previous studies (1,11) reporting that the exon-deleted ER variants ER Δ E2, ER Δ E3, and ER Δ E7 fail to stimulate pERE-TK-CAT reporter plasmid expression and add ER Δ E4 and the hypothetical ER Δ E6 mutant to the list of transcriptionally deficient splicing variants. These new observations on the behavior of ER Δ E4 represent the first functional characterization of this variant and are significant in light of the recently reported widespread occurrence of the ER Δ E4 variant in human breast tumors (12). While these experiments argue fairly conclusively that ER splicing variants other than ER Δ E5 appear to be devoid of *positive* transcriptional activity, they leave open the question of potential *negative* or dominant inhibitory transcriptional activity, as we have previously reported for the ER Δ E3 variant (1). Additional experiments involving cotransfection of each of the splicing variants along with wt ER and an estrogen-responsive reporter plasmid (pERE-TK-CAT) are in progress to address this issue.

As noted in a previous progress report, ER Δ E5 appears to be unique among the ER splicing variants in being able to support a limited degree of residual transcriptional activation. Since this mutant harbors a frame-shift mutation that prematurely truncates translation of this receptor within the ligand-binding domain (Appendix B), ER Δ E5 is unable to bind estrogen and its transcriptional activity is ligand-independent. This phenomenon was originally described by Fuqua and colleagues (13) who analyzed the expression of ER Δ E5 in a heterologous gene expression in the yeast (*S. cerevisiae*). The constitutive transcriptional activity of ER Δ E5 is cell type-dependent and presumably requires a cellular environment in which induction of gene expression by ER is dominated by the amino terminal activation function (AF1) that remains intact in this mutated receptor. Fig. 2 shows that chicken embryo fibroblasts (CEF cells) represent one such

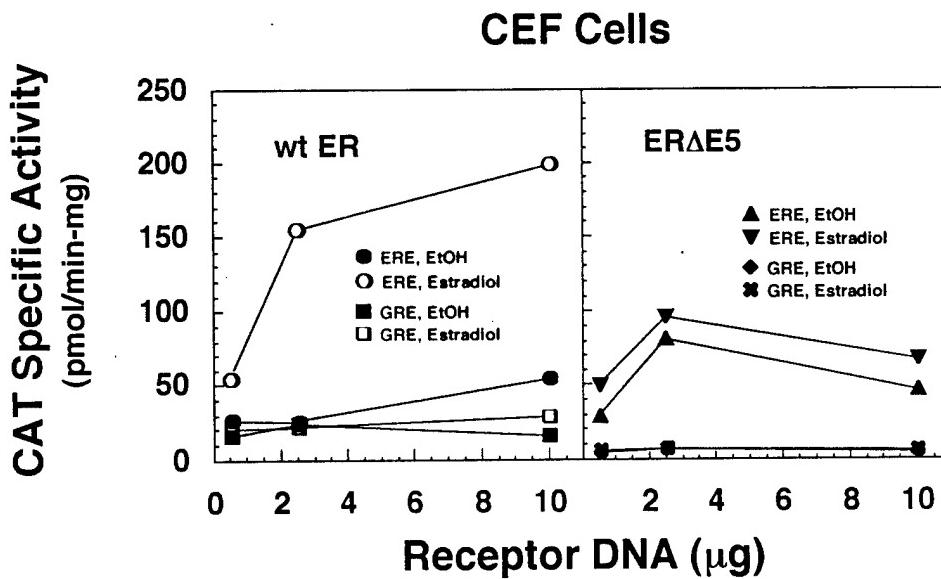


Fig. 2. Titration of transcriptional activity of ER Δ E5 vs wt ER in CEF cells.

environment, consistent with the studies of Tora *et al.* (14) that demonstrated the AF1 dominance of CEF cells. In contrast to the wt ER, induction of transcription by ERAE5 in CEF cells is unaffected by hormone. Notably, ERAE5 supported CAT enzyme expression on an ERE-containing, but not a GRE-containing reporter plasmid (Fig. 2), indicating that DNA binding appears to be required for transcriptional activation by this variant.

Our previous progress report included data showing that ERAE5 also appears to possess modest transcriptional activity in MDA MB231 cells, an ER-negative breast tumor cell line. In this cell line, ERAE5 increased basal activity of the pERE-TK-CAT reporter plasmid 2.5-fold higher than the background level supported by the pCMV4 vector alone ($P<0.005$). While this may suggest that breast-derived cell lines in general display AF-1 dependence, the significance of these observations is unclear since mRNA analysis using a sensitive PCR assay failed to detect the presence of any ER transcripts in this cell line (3). In contrast, HeLa and Cos7 cells which are also ER-negative, failed to support any measurable transcriptional activation in response to ERAE5. These findings are consistent with a recent report characterizing the behavior of the ERAE5 splicing variant in transiently and stably transfected cells (15).

Estrogen-independent activation of the ERAE5 splicing variant.

The apparent dependence of ERAE5 on AF-1 function raises the important question of whether or not the transcriptional activity of this variant can be regulated by ligand-independent modulators of ER activity. The current view is that AF-1 of ER is comprised in part of phosphorylation sites for ser/thr-dependent protein kinases such as MAPK, Casein Kinase II, and perhaps protein kinase A. Phosphorylation of critical residues by these kinases presumably permits this receptor to interact more efficiently with other components of the transcriptional machinery. A variety of inducers other than estrogens (e.g., growth factors, phorbol esters, cAMP, and dopamine) have been shown to stimulate ER activity in the absence of ligand, or to synergize with estradiol to augment gene expression. It is presumed that these inducers activate signal transduction cascades that result in altered phosphorylation of ER, or other transcription factors that cooperate with ER to activate gene expression. We are currently reassessing the behavior of ERAE5 in view of these recent findings to determine if ligand-independent modulators of ER function, in particular growth factors, alter the transcriptional activity of this splicing variant. A related series of experiments are planned to determine whether known coactivators of ER function (e.g., SRC-1 and CBP) have any effect on the behavior of ERAE5.

Measurement of the DNA-binding activity of the ER splicing variants.

Efficient DNA-binding by ER requires the cooperation of several functional elements within this protein, including the centrally located DNA-binding domain itself and a ligand-inducible subunit dimerization motif located towards the end of the hormone-binding region (16). It is also possible that other subunit contacts occur elsewhere in the protein. Because all of the ER splicing variants have sustained deletions within various regions of this protein, it was of interest to systematically assess the DNA-binding ability of each variant. For this purpose, gel mobility shift assays were performed using extracts prepared from transfected Cos7 cells incubated with an oligonucleotide containing a consensus estrogen response element (AGGTCACAGTGACCT). As expected, variants that harbor a mutation within the DNA-binding domain (ERAE2 and ERAE3) have lost all potential to recognize the appropriate DNA response element (Fig. 3, lanes 5-8). More surprisingly, the loss of exons contributing to the ligand-binding domain also result in a strong defect in DNA recognition (lanes 9-16). For ERAE5 and ERAE7, however, this appears to be a quantitative defect in DNA-binding, rather than a qualitative change, since the addition of an ER-specific monoclonal antibody (Mab-17) to the binding reactions results in the recovery of weak DNA binding (lanes 12 and 16), due presumably to the ability of the bivalent antibody to stabilize

the interaction of two receptor subunits to their palindromic binding site. This observation resolves the apparent contradiction between the inability of $\text{ER}^{\Delta E5}$ to bind to an ERE even though it appears to be capable of weakly activating transcription through this response element. These data also reconfirm the importance for DNA binding of the dimerization motif located within exon 7 of the ER sequence.

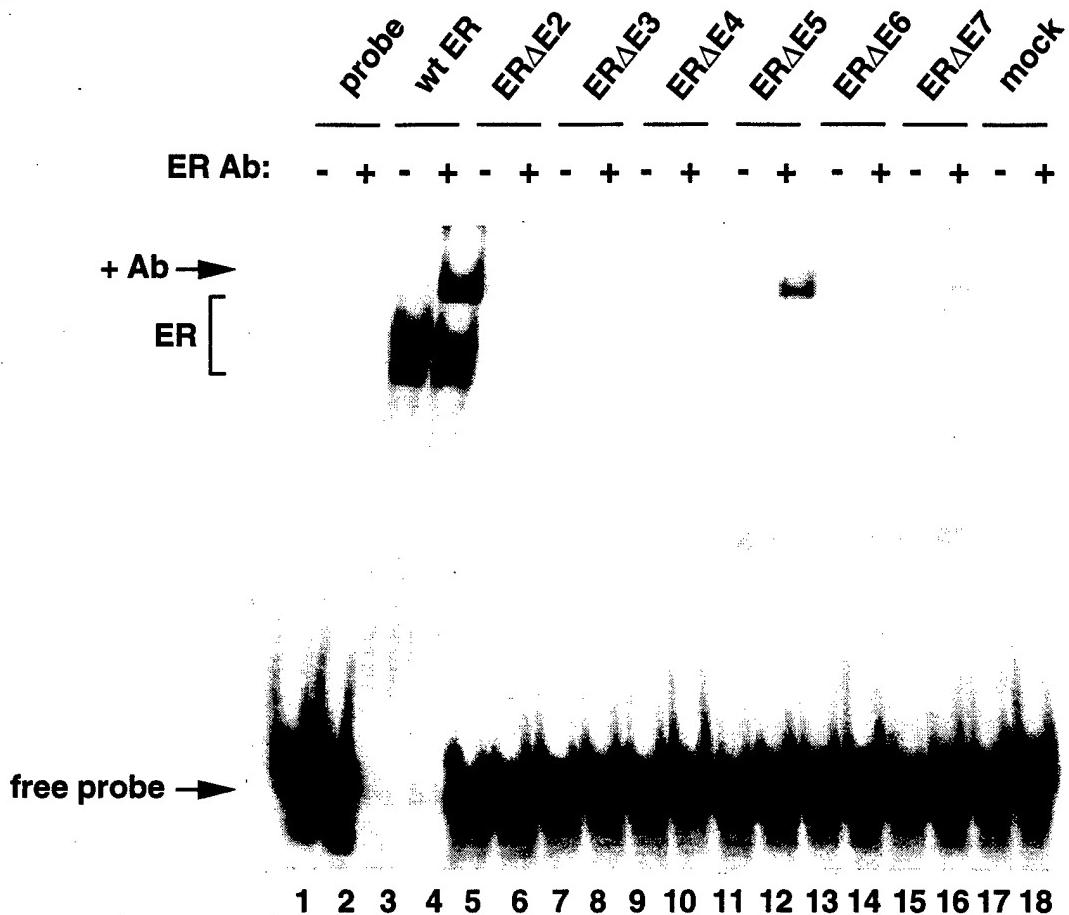


Fig 3. DNA-binding activity of ER splicing variants in the absence and presence of ER-antibody.

The presence of comparable amounts of wt ER and variant receptors in these extracts was confirmed by immunoblot analysis using the Mab-17 antibody. Such blots furthermore demonstrate that each of these ER splicing variants appears to represent a stable protein able to accumulate to significant levels within expressing cell lines. This represents an important new finding since essentially all of the studies reported to date on these variants have generally involved frequently use of the PCR technique to analyze expression at the level of RNA.

Subcellular localization of ER splicing variants.

Having developed efficient vectors for expressing a number of the ER mRNA splicing variants as well as antibodies for their detection, we've also undertaken an analysis of the subcellular distribution of these variant receptor proteins. For our studies Cos7 cells were transiently transfected with pCMV4 or pcDNA3.1 vectors harboring cDNAs encoding wt or variant receptors. These receptors were detected in transfected cells by indirect immunofluorescence using the Mab-17 ER antibody, as described in Neff *et al.* (10). Both ERAE3 and ERAE5 localize almost entirely to the nuclei of transfected cells, similar to the behavior of wt ER (data not shown). This result is consistent with the fact that each of these receptors retains the tripartite karyophilic signal just downstream of the DNA-binding domain (17). The nuclear distribution of ERAE3 is also consistent with its ability to interfere with the transcriptional stimulatory activity of wt ER (1). The only notable difference observed between ERAE5 and wt ER or ERAE3 is that ERAE5 displays a pronounced perinuclear "halo" of receptor, that may reflect a slower rate of nuclear uptake compared with wt ER. A block in the nuclear uptake of ERAE5 can not, however, account for its dramatically impaired transcriptional stimulatory activity, since the majority of ERAE5 readily accumulates within the nucleus of transfected cells. Subcellular localization studies have also been completed for the ERAE2, ERAE4, ERAE6, and ERAE7 variants. Each of these proteins can be readily detected with transfected cells, but they all possess dramatic defects in nuclear targeting. Nuclear targeting of ER is governed almost exclusively by a tripartite karyophilic signal present within exon 4. Loss of this signal is therefore consistent with the cytoplasmic pattern of distribution of mutants such as ERAE2 and ERAE4, both of which lack exon 4 sequences (Appendix B). Inappropriate presentation of this signal must, however, account for the defects in nuclear localization seen with ERAE6 and ERAE7 since the karyophilic signal is entirely spared in these latter two variants.

DISCUSSION

Shortly following the approval of this award, a request for a change in the performance site of this project was filed, necessitated by a change in the institutional affiliation of the PI (R. Miksicek). This was accomplished by a research subcontract agreed upon by the two institutions involved (The Research Foundation of SUNY and Michigan State University) and approved by the US Army MRMC. Despite the disruptions caused by this change in performance site and the hiring and training of new research personnel at MSU, significant progress has still been made during the first two years of this award towards reaching the specific aims set out in our original application. This progress was substantially hampered between 07/01/95 and 03/28/96, during which time terms of the research subcontract were being negotiated. Funds for the payment of salaries and research expenses were finally released at MSU on 03/28/96, permitting resumption of work.

The major accomplishments for this reporting period include completion of the cloning and construction of plasmids for the individual expression of ER splicing variants and characterization of their behavior as mutated transcription factors. When tested in a transient transfection system, all six of these splicing variants shows efficient expression, and each displays a characteristic pattern of subcellular distribution that can be distinguished from that of intact receptor. Each of the variants also displays a dramatic reduction in DNA-binding activity, as measured using a gel mobility shift assay *in vitro*. While this DNA-binding defect appears to be complete for ERΔE2, ERΔE3, ERΔE4, and ERΔE6 weak residual DNA binding is discernible for ERΔE5 and ERΔE7 when DNA complex formation is stabilized by the addition of a bivalent antibody capable of recognizing the ER protein. We tentatively conclude that there are two causal factors involved in the loss of efficient DNA-binding by these receptor variants: 1) absence of a crucial dimerization element that has been mapped to the carboxy-terminal end of the hormone-binding domain of ER (16) and 2) disruption in the structure or accessibility of the "Zinc-finger" DNA-binding domain caused by remnants of the hormone-binding domain still present in many of the variants. Activities that remain to be assessed for the splicing variants includes a measurement of their ligand-binding affinities, determination of their abilities to respond to ligand-independent activators of ER such as growth factors, protein kinase activators, and nuclear receptor coactivators (SRC-1 and CBP/P300), and an evaluation of their potential for interfering with the function of co-expressed wt ER. A manuscript is in preparation describing our biochemical characterization of ER splicing variants for submission early in 1997.

The ERΔE5 variant has attracted special interest due to an early report (13) suggesting that this isoform may possess constitutive transcriptional activity that could account for some aspects of tamoxifen resistance, a common problem in the clinical treatment of breast cancer. Our findings indicate that the transcriptional activity of ERΔE5 is relatively weak and is highly cell-type specific, indicating that the common perception of ERΔE5 as an "outlaw" receptor needs to be re-evaluated, as recently suggested by Rea and Parker (19). Further work analyzing the behavior of ERΔE5 in stably and transiently transfected cell lines is in progress to clarify this issue. A manuscript describing the transcriptional behavior of ERΔE5 and reporting on the production of an ERΔE5-specific monoclonal antibody is in preparation for submission during 1997.

During the final years of this project, greater emphasis will be placed on the use of PCR amplification to quantitatively assess the pattern of expression of ER splicing variants in breast tumors. Conditions have now been established using 3'-mismatched primer pairs to individually amplify and quantitate correctly and aberrantly processed receptor transcripts. This work will focus on analyzing the extent of aberrant splicing in ER mRNA, but will also include the limited analysis of the PR message, as proposed in the original specific aims for this project.

Publications From Current Award Year:

1) publications related to this award

- a) Bunone, G., Briand, P.-A., **Miksicek, R.J.**, and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.*, 15(9):2174-2183.
- b) Elias, J.M., Hyder, D.M., **Miksicek, R.J.**, Heimann, A., and Margiotta, M. (1995) Interpretation of steroid receptors in breast cancer: a case with discordant estrogen receptor results using ER1D5 and H222. *J. Histotechnology*, 18(4):331-335.

2) publications unrelated to this award

- a) Scarlata, S. and **Miksicek, R.** (1995) Binding properties of coumestrol to expressed human estrogen receptor. *Mol. Cell. Endocrinol.*, 115:65-72.
- b) **Miksicek, R.J.**, Lee, C., Topp-Rapiejko, K., and Neff, S. (1995) Interference with nuclear transport is not essential for inhibition of receptor function by the type II estrogen antagonist ICI-164-384. *Ann. N.Y. Acad. Sci.*, 761:361-364.

3) invited articles

- a) **Miksicek, R.J.** (1996) The utility of fluorescent estrogens for receptor imaging, in *Estrogens, Progestins, and their Antagonists*, E. Pavlik, ed., Birkhäuser, Boston, pp 245-273.
- b) **Miksicek, R.J.** and Katzenellenbogen, J.A. (1996) Estrogen receptor imaging using intrinsically fluorescent ligands, in *Analytical Use of Fluorescent Probes in Oncology*, Plenum Press, New York, pp 213-219.

REFERENCES

- 1) Wang, Y. and Miksicek, R.J. (1991) Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.* **5**:1707-1715.
- 2) Fuqua, S.A.W., Chamness, G.C. and McGuire, W.L. (1993) Estrogen receptor mutations in breast cancer. *J. Cell. Biochem.* **51**:135-139.
- 3) Miksicek, R.J., Lei, Y., and Wang, Y. (1993) Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. *Breast Cancer Res. and Treatment*, **26**:163-174.
- 4) Gotteland, M., Desauty, G., Delarue, J.C., Liu, L., and May, E. (1995) Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. *Mol. Cell. Endocrinol.* **112**:1-13.
- 5) Fuqua, S.A.W., Wolf, D.M. (1995) Molecular aspects of estrogen receptor variants in breast cancer. *Breast Cancer Res. and Treatment*, **35**:233-241.
- 6) Miksicek, R.J. (1994) Steroid receptor variants and their potential role in cancer. *Seminars in Cancer Biology* **5**:369-379.
- 7) Sluyser, M. (1995) Mutations in the estrogen receptor gene. *Human Mutations* **6**:97-103.
- 8) Andersson, S., Davis, D.N., Dahlback, H., Jornvall, H., and Russell, D.W. (1989) Cloning, structure, and expression of the mitochondrial cytochrome P450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol Chem.* **264**:8222-8229.
- 9) Miksicek, R.J., Carlson, K.E., Hwang, K.-J., and Katzenellenbogen, J.A. (1995) Studies using fluorescent tetrahydrochrysene estrogens for *in situ* visualization of the estrogen receptor in living cells. *Mol. Endocrinol.* **9**:592-604.
- 10) Neff, S., Sadowski, C., and Miksicek, R.J. (1993) Site-directed mutagenesis of the hormone-binding domain of the human estrogen receptor reveals that two cysteine residues play a role in DNA-binding and subcellular localization. *Mol. Endocrinol.* **8**:1215-1223.
- 11) McGuire, W.L., Chamness, G.C., and Fuqua, S.A.W. (1991) Estrogen receptor variants in clinical breast cancer. *Mol. Endocrinol.* **5**:1571-1577.
- 12) Leygue, E., Huang, A., Murphy, L.C., Watson, P.H. (1996) Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. *Cancer Res.* **56**:4324-4327.
- 13) Fuqua, S.A.W., Fitzgerald, S.D., Chamness, G.C., Tandon, A.K., McDonnell, D.P., Nawaz, Z., O'Malley, B.W., and McGuire, W.L. (1991) Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.* **51**:105-109.
- 14) Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., Chambon, P. (1989) The human estrogen receptor has two independent nonacidic transcriptional activation functions *Cell* **59**:477-487.
- 15) Rea, D., Parker, M.G. (1996) Effects of an exon 5 variant of the estrogen receptor in MCF7 breast cancer cells. *Cancer Res.* **56**:1556-1563.
- 16) Lees, J.A., Fawell, S.E., White, R., Parker, M.G. (1990) A 22-amino acid peptide restores DNA-binding activity to dimerization defective mutants of the estrogen receptor. *Mol. Cell. Biol.* **10**:5529-5539.
- 17) Ylikomi, T., Bocquel, M.T., Berry, M., and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.* **11**:3681-3694.

Appendix A: Sequence of Oligodeoxynucleotides Obtained for this Project

1)	ERΔE4 Upper	5' - TTGTCGACTTGACCCTCCATGATCAGGT -3'	28 mer
2)	ERΔE4 Lower	5' - AAGTCGACAAAGCCACCTTCATCATTCAC -3'	32 mer
3)	ERΔE6 Upper	5' - GGATCGATCTGTACACATTCTGTCCAGC -3'	29 mer
4)	ERΔE6 Lower	5' - CAGATCGATCCAAGAGCAAGTTAGGAGC -3'	28 mer
5)	E3Lwt	5' - CACCTTCATCATTCCCACT -3'	20 mer
6)	E4Lwt	5' - ATACTCGGAATAGAGTATGG -3'	20 mer
7)	E5Lwt	5' - CTGTCCAAGAGCAAGTTAGG -3'	20 mer
8)	E6Lwt	5' - GCAAAATAATAGATTTGAGG -3'	20 mer
9)	E7Lwt	5' - CTCATGTGCCTGATGTGGGA -3'	20 mer
10)	E8Lwt	5' - CAAGGAATGCGATGAAGTAG -3'	20 mer
11)	E1Uwt	5' - CGCCGGCATTCTACAGGC -3'	18 mer
12)	E1UΔE2	5' - CGCCGGCATTCTACAGGA -3'	18 mer
13)	E1UΔE2/3	5' - CGCCGGCATTCTACAGGG -3'	18 mer
14)	E2Uwt	5' - TCAAGAGAAGTATTCAAGGA -3'	20 mer
15)	E2UΔE3	5' - TCAAGAGAAGTATTCAAGGG -3'	20 mer
16)	E2UΔE3/4	5' - TCAAGAGAAGTATTCAAGGC -3'	20 mer
17)	E3Uwt	5' - TGGGAATGATGAAAGGTGGG -3'	20 mer
18)	E3UΔE4	5' - TGGGAATGATGAAAGGTGGC -3'	20 mer
19)	E3UΔE4/5	5' - TGGGAATGATGAAAGGTGGA -3'	20 mer
20)	E4Uwt	5' - GAAGAGGGTGCCAGGCT -3'	17 mer
21)	E4UΔE5	5' - GAAGAGGGTGCCAGGAA -3'	17 mer
22)	E4UΔE5/6	5' - GAAGAGGGTGCCAGGAG -3'	17 mer
23)	E5Uwt	5' - AAC TTGCTCTTGGACAGGAA -3'	20 mer
24)	E5UΔE6	5' - AAC TTGCTCTTGGACAGGAG -3'	20 mer
25)	E5UΔE6/7	5' - AAC TTGCTCTTGGACAGT -3'	18 mer
26)	E6Uwt	5' - CTATTATTTGCTTAATTCTGG -3'	22 mer
27)	E6UΔE7	5' - CTATTATTTGCTTAATTCTGT -3'	22 mer

Appendix B: Predicted Structures of Exon-Skipped ER Splicing Variants

